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Title: Nalidixic Acid Resistance Increases Sensitivity of *Escherichia coli* O157:H7 to
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Nalidixic Acid Resistance Increases Sensitivity of *Escherichia coli* O157:H7 to Ionizing Radiation in Solution and on Green Leaf Lettuce

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ABSTRACT: Nalidixic acid resistance has been used as a selective marker for studies of pathogen-inoculated fruits and vegetables. Three nalidixic acid-sensitive outbreak strains of *Escherichia coli* O157:H7 were used to generate mutants resistant to nalidixic acid (Nal^R, 50 µg/mL) by successive culturing and selection in nalidixic acid-amended broth. The resistance to ionizing radiation of the parent and Nal^R strains was determined (a) in a phosphate buffer solution and (b) on green leaf lettuce. The Nal^R strains of each of the 3 isolates were significantly ($P < 0.05$) more sensitive to ionizing radiation than the nalidixic acid-sensitive (Nal^S) parent strains in both systems. D_{10} values (the amount of ionizing radiation required to achieve 1 log₁₀ reduction) determined in buffer for the parent strains ranged from 0.18 to 0.33 kGy, whereas for the Nal^R strains, D_{10} were approximately 0.10 kGy, a reduction of up to 69%. When evaluated on green leaf lettuce, the D_{10} for the Nal^S strains was approximately 0.18 kGy as opposed to 0.10 to 0.12 kGy for the Nal^R strains, a reduction of up to 45%. The D_{10} values obtained on lettuce were significantly different than those obtained in buffer for 4 of the 6 isolates examined. The magnitude of the increase in radiation sensitivity resulting from resistance to nalidixic acid varied among the strains tested and also varied depending on the suspending medium. These results suggest that the use of nalidixic acid resistance as a selective marker may result in significant overestimates of the antimicrobial efficacy of ionizing radiation against *E. coli* O157:H7.

Keywords: irradiation, gamma, D value, radiation pasteurization, *Lactuca sativa*, antibiotic resistance

Introduction

Fresh and fresh-cut fruits and vegetables can be contaminated with human pathogens such as *Escherichia coli* O157:H7, resulting in illness (Tauxe and others 1997; Gombas and others 2003; Horry and others 2003). Studies of interventions to remove or inactivate human pathogens are complicated by the relatively high level of background microflora that fresh produce typically contains (Niemira 2003). In studies of inoculated produce, selective media are able to distinguish pathogens of interest from background microflora; however, selective media may not support the growth of bacteria injured by the antimicrobial intervention (Ray 1979). An alternative is the use of antibiotic-resistant pathogen strains and antibiotic-amended media in inoculation studies (Blackburn and Davies 1994). A key factor in the use of this approach is validation, the confirmation that the antibiotic resistant strains accurately reflect the response of the original antibiotic-sensitive parent strain to the intervention being tested. Resistance to the quinolone antibiotic nalidixic acid was validated for use as a marker in studies of chemical interventions and growth parameter measurements (Blackburn and Davies 1994; Taormina and Beuchat 1999). In these studies, it was demonstrated that nalidixic acid-resistant strains had similar growth rates, stress tolerances, and so forth, as nalidixic acid-sensitive parent strains. However, no such validation has been offered for use of this selective marker for use with studies of ionizing radiation.

Low doses of ionizing radiation effectively reduce the level of pathogenic bacteria on fresh produce (Foley and others 2002; Niemira and others 2002; Niemira 2003). As the radiation sensitivity of a given isolate can be significantly different based on the type of subtending vegetable examined (Niemira and others 2002), the D_{10} value (the radiation dose required to reduce the pathogen population by 1 log₁₀ unit, or 90%) for a given pathogen should be determined on the intended product. Overestimation of the D_{10} value may lead to the recommendation of unnecessarily high doses. Of more concern from a food safety standpoint, underestimation of the radiation D_{10} value may lead to inadequate control of the target pathogen by the irradiation process.

This study was done to validate the use of nalidixic acid-resistant strains of *E. coli* O157:H7 in studies of the efficacy of ionizing radiation using model solutions and inoculated green leaf lettuce.

Materials and Methods

Microorganisms

Three strains of *E. coli* O157:H7 were maintained in tryptic soy broth (TSB, Difco, Detroit, Mich., U.S.A.): C9490 (Centers for Disease Control, Atlanta, Ga., U.S.A.), ATCC-35150, and ATCC-43894 (American Type Culture Collection, Manassas, Va., U.S.A.). The 3 strains were determined to be sensitive to a level of 50 µg/mL nalidixic acid (Sigma Chemical Co., St. Louis, Mo., U.S.A.) in amended TSB. The nalidixic acid-sensitive (Nal^S) parent strains were designated C9490-S, 35150-S, and 43894-S. Subcultures of these strains were adapted to nalidixic acid after the method of Taormina and Beuchat (1999). Solutions (5 mL) of sterile TSB were amended to 5, 10, 20, 30, 40, and 50 µg/mL using sterile filtered (0.22 µm) na-

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lidixic acid stock solutions. Every 24 h over a period of 6 d, 200 μ L of culture was taken from the lower concentration solution and used to inoculate the next higher concentration solution. Cultures were grown at 37 °C in a shaking incubator, 150 rpm. The nalidixic acid-resistant (*Nal*^R) daughter strains were designated C9490-R, 35150-R, and 43894-R. The cell concentration in overnight cultures (37 °C) of *Nal*^S and *Nal*^R strains was determined to be about 10⁹ colony-forming units (CFU)/mL.

*D*₁₀ in solution

Nal^S and *Nal*^R strains were grown overnight (37 °C) in 10-mL tubes of TSB. The cultures were centrifuged (5000 × *g*) to pelletize the cells. The cells were resuspended in 10-mL aliquots of Butterfield's phosphate buffer (BPB, Applied Research Inst., Newtown, Conn., U.S.A.). *Nal*^S and *Nal*^R strains had similar growth patterns in overnight culture, and a final concentration of about 10⁸ CFU/mL. To more clearly delineate the effects of antibiotic resistance, the cultures were evaluated as individual isolates, rather than as a multi-isolate cocktail. The tubes were held at 4 °C until irradiation, typically 30 to 60 min.

To determine the effect of exposure to nalidixic acid on the *Nal*^R strains immediately before the irradiation process, the radiation *D*₁₀ value of the *Nal*^R strains was also determined after growth in nalidixic acid-amended TSB (50 μ g/mL). These cells were centrifuged and resuspended in BPB as previously described.

To determine the effect of exposure to nalidixic acid on the *Nal*^R strains immediately after the irradiation process, the *Nal*^R strains were grown in unamended TSB, centrifuged, and resuspended in BPB as described. Immediately after irradiation, the tubes were amended with stock solutions of nalidixic acid to make a final concentration of 50 μ g/mL, and vortexed. The tubes were held on the benchtop for about 1 h before diluting and plating (described subsequently).

*D*₁₀ on green leaf lettuce

Fresh green leaf lettuce was obtained from local markets on the day of each experiment. The outer leaves and any obviously damaged leaves of each head were removed and discarded. Cut leaf pieces were prepared, sanitized with a 300-ppm sodium hypochlorite solution, rinsed 5 times with sterile distilled water, and inoculated separately with the individual *Nal*^S and *Nal*^R strains according to the method of Niemira and others (2002). Briefly, the sanitized cut leaf pieces were completely submerged in inoculum (approximately 10⁸ CFU/mL for each of the isolates) for 120 s and then transferred to a sterile salad spinner-type centrifuge. The material was spun twice to remove excess inoculum from the surface of the leaf pieces. Samples (45 g) of each lettuce type were placed in nr 400 Stomacher bags (Tekmar, Inc., Cincinnati, Ohio, U.S.A.). The procedures were performed in a bio-safety cabinet. The samples were refrigerated (4 °C) until irradiation, typically 30 to 60 min.

Irradiation

The inoculated BPB solutions or the inoculated leaf pieces were treated with 0.0 (control), 0.1, 0.2, 0.3, 0.4, or 0.5 kGy. In all cases, the irradiation was conducted at 4 °C. Temperature control was maintained during irradiation by injection of gas coming from liquid nitrogen into the sample chamber. The samples were irradiated using a Lockheed-Georgia (Marietta, Ga., U.S.A.) cesium-137 self-contained gamma radiation source, with a dose rate of 5.56 kGy/h. The dose rate was established using alanine transfer dosimeters from the National Institutes of Standards and Technology (Gaithersburg, Md., U.S.A.). Alanine pellets (Bruker, Inc. Billerica, Mass., U.S.A.) were used for dosimetry. The pellets were read on a Bruker EMS 104 EPR analyzer

Table 1 — Radiation resistance of nalidixic acid-sensitive and nalidixic acid-resistant strains of *Escherichia coli* O157:H7

Isolate ^a	<i>D</i> ₁₀ in buffer (kGy)	<i>R</i> ²	5 log ₁₀ dose (kGy)	<i>D</i> ₁₀ on lettuce (kGy)	5 log ₁₀ dose <i>R</i> ²	Suspending medium <i>P</i> value
C9490-S	0.180a ^b	0.98	0.900 ^c	.181a	0.94	0.903
C9490-R	0.099d	0.99	0.495	.101b	0.97	0.503
35150-S	0.334b	0.96	1.670	.185a	0.92	0.927
35150-R	0.103de	0.99	0.515	.122c	0.95	0.609
43894-S	0.251c	0.95	1.255	.182a	0.96	0.910
43894-R	0.099e	0.99	0.495	.105b	0.98	0.526

^aIsolates of are sensitive to (suffix: "S") or resistant to (suffix: "R") nalidixic acid, 50 μ g/mL.

^b*D*₁₀ values in a given suspending medium (column) followed by the same letter are not significantly different (analysis of covariance, *P* < 0.05).

^cDose required to achieve reduction of 5 log₁₀ colony-forming units (CFU)/mL units, based on the preceding *D*₁₀ values.

^dNSD = *D*₁₀ values for a given isolate (row) are not significantly different (analysis of covariance, *P* < 0.05).

and compared with a previously determined standard curve. Actual dose was typically within 5% of the nominal dose.

Sampling

After irradiation, the solutions were immediately serially diluted with sterile BPB, except for the solutions used to evaluate the effects of postirradiation exposure to nalidixic acid, which were allowed to sit for about 1 h, as described previously. After dilution, 1-mL samples were taken and pour-plated with tryptic soy agar (TSA, Difco, Detroit, Mich., U.S.A.). Three pour-plates per dilution were incubated at 37 °C for 24 h and counted with a calibrated AccuCount 1000 automated colony counter (Biologics, Gainesville, Va., U.S.A.).

After irradiation, the cut leaf samples were refrigerated until microbiological sampling, typically 60 to 90 min. Sterile BPB (180 mL) was added to the stomacher bag and agitated for 60 s. A 1-mL sample was withdrawn for serial dilution with sterile BPB and pour-plating with TSA. The plates were incubated and counted as described.

The data for each sample were normalized against the control and plotted as the log₁₀ reduction using the nominal doses. Each experiment was performed 3 times and the data pooled. The slopes of the individual survivor curves were calculated with linear regression (SigmaPlot 5.0, SPSS Inc., Chicago, Ill., U.S.A.) and compared using analysis of covariance (ANCOVA, Excel 97, Microsoft Corp, Redmond, Wash., U.S.A.). The ionizing radiation *D*₁₀ value was calculated by taking the negative reciprocal of the survivor curve slope (QuattroPro, Corel Corp., Ottawa, Ontario, Canada).

Results and Discussion

When treated in buffer solutions, *Nal*^R derivative strains were significantly (*P* < 0.05) more sensitive to ionizing radiation than *Nal*^S parent strains (Figure 1). *D*₁₀ values were reduced by 45% to 69% in a strain-dependent manner (Table 1). Growth in non-amended TSB, TSB amended with 50 μ g/mL nalidixic acid, and exposure to 50 μ g/mL nalidixic acid immediately after irradiation had no effect on the radiation sensitivity of *Nal*^R strains (Figure 2).

When treated on the surface of green leaf lettuce, *Nal*^R derivative strains were significantly (*P* < 0.05) more sensitive to ionizing radiation than *Nal*^S parent strains (Figure 3). *D*₁₀ values were reduced by 35% to 45% in a strain-dependent manner (Table 1).

Overall, the *D*₁₀ values obtained on green leaf lettuce were variable relative to those obtained in buffer, and were isolate-dependent in their response (Table 1). *D*₁₀ values were significantly (*P* < 0.05) decreased (35150-S, 43894-S), significantly increased (35150-R, 43894-R), or not significantly different (C9490-S, C9490-R).

Discussion

The results presented herein demonstrate that strains of *E. coli* O157:H7 that have been rendered resistant to nalidixic acid are also rendered more sensitive to ionizing radiation. The extent to which the radiation sensitivity is increased is isolate-specific and strongly influenced by the medium used for the evaluation. Nalidixic acid and related quinolone antibacterial agents act by inhibiting DNA synthesis and replication by interfering with the action of DNA gyrase and topoisomerase IV (Georgopapadakou and others 1987; Khadursky and Cozzarelli 1998). The quinolone group is not known to significantly interfere with DNA repair. In contrast, the primary mode of action of ionizing radiation is via hydrogen and hydroxyl radical molecules resulting from the ionization of water molecules within the target; these radicals disrupt membranes, interfere with the functioning of proteins, and can lead to strand breakage of DNA (Niemira 2003).

The effects of nalidixic acid are therefore seen as abnormal DNA synthesis and replication, rather than in DNA cleavage and subsequent repair as with radiation damage. The disparate nature of the mechanisms of action of these 2 processes do not readily suggest a means by which they may be interrelated. Growth in nalidixic acid-amended versus non-amended TSB or exposure to nalidixic acid immediately after irradiation had no significant effect on D_{10} values obtained. This suggests that the biochemical mechanism(s) that the *Nal*^R isolates use to mitigate or nullify the effects of nalidixic acid is not influenced by the irradiation treatment. Irradiation can result in double strand breaks in DNA and interstrand cross-links, which are repaired by homologous recombination in *E. coli* (Kornberg and Baker 1992). The RecBCD pathway is primarily responsible for *E. coli* conjugation and recombination and requires a fully functional DNA gyrase to operate properly. Exposure to chemical stressors such as hydrogen peroxide and the DNA cross-linking agent mitomycin C increased the expression of the *gyrA* promoter fused

to a *lacZ* gene (Orser and others 1995), suggesting the possibility that radiation stress may lead to a similar induction that has a synergistic interaction with the *Nal*^R mechanism. However, the precise mechanism(s) of the resistance to nalidixic acid in the *Nal*^R isolates used herein has not been characterized on a molecular level. This resistance may result from a mutated *gyrA* gene, which codes for a nalidixic acid nonsensitive mutated form of the DNA gyrase, a novel metabolic degradation pathway of nalidixic acid, modulation of the activity of nalidixic acid within the cells, or some other process. The possibility also exists that the selection process used to generate the *Nal*^R mutants may have also resulted in additional, unidentified, mutations, which may exert an influence on radiation sensitivity. As the *Nal*^R mutants were generated by undirected selection, rather than as the result of the insertion of a defined genetic construct, or deletion of an identified section of native DNA related to nalidixic acid sensitivity, it is entirely likely that the specific form of resistance differs from isolate to isolate. A clearer understanding of how the cells protect the native DNA gyrase from the action of nalidixic acid may elucidate the mechanism responsible for the demonstrated increase in sensitivity to irradiation.

The extent of the interrelationship of antibiotic resistance and resistance to ionizing radiation is not known. Nalidixic acid-resistant mutant strains of *Salmonella*, generated from nalidixic acid-sensitive parent strains, show a similarly increased sensitivity to ionizing radiation as that shown herein with *E. coli* O157:H7 (B.A. Niemira, unpublished data). Resistance to 1 antibiotic may result in a certain level of cross-protection against multiple different antibiotics. Golding and Matthews (2004) showed that the vast majority of 52 strains of *E. coli* O157:H7, induced to be chloramphenicol resistant, were subsequently shown to have an increased tolerance to tetracycline, ciprofloxacin, and nalidixic acid. The potential sig-

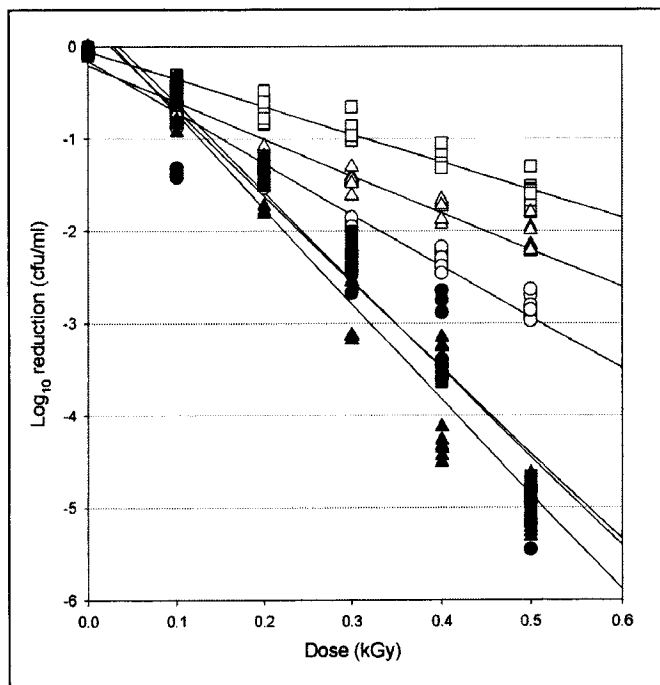


Figure 1—Radiation sensitivity of nalidixic acid sensitive (*Nal*^S, white) and resistant (*Nal*^R, black) isolates of *Escherichia coli* O157:H7 in a model buffer solution. Isolates C9490-F (circle), 35150-R (square), and 43894 (triangle) were grown in unamended tryptic soy broth (TSB).

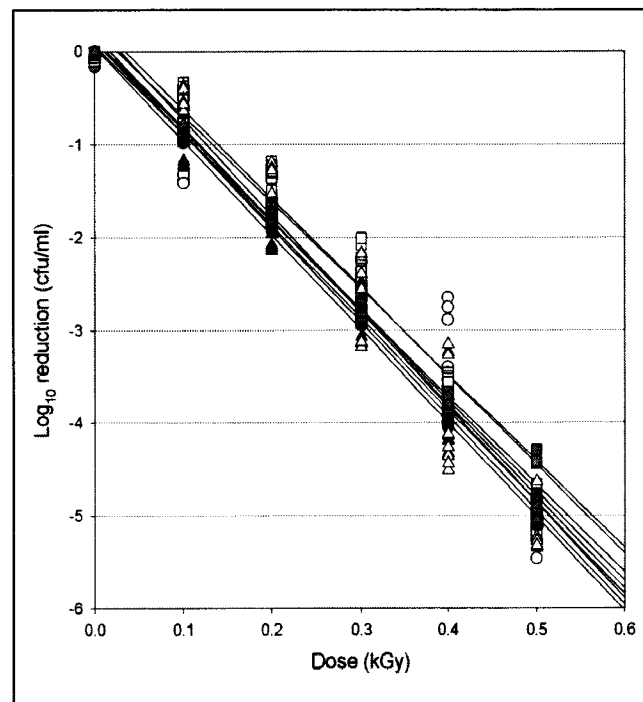


Figure 2—Radiation sensitivity of nalidixic acid-resistant isolates of *Escherichia coli* O157:H7. Isolates C9490-F (circle), 35150-R (square), and 43894-R (triangle) were grown in unamended tryptic soy broth (TSB) (black) or TSB amended with 50 µg/mL nalidixic acid (gray) before irradiation, or exposed to 50 µg/mL nalidixic acid immediately after irradiation (white).

nificance of this cross-protection with regard to the efficacy of antimicrobial interventions such as irradiation is uncertain, particularly in the context of food substrates. Data are not available on the relative radiation resistance of isolates that are resistant to chloramphenicol, tetracycline, ciprofloxacin, or other antibiotic agents. As the concerns related to antibiotic resistant pathogens increase (Golding and Matthews 2004), research that more fully explores the relative susceptibility of these resistant isolates to antimicrobial interventions become more important. It may be speculated that antibiotic-resistant contaminating pathogens may be more amenable to elimination by ionizing radiation, thereby enhancing the cumulative efficacy of the process in a food-safety context. However, in the absence of extensive data, the isolate-specific nature of the change in radiation sensitivity makes such speculation problematic. Additional research on the radiation sensitivity of additional antibiotic-resistant pathogen strains, in model systems and on products, will serve to improve understanding of the relationship between these phenomena.

Conclusions

The results presented herein have important implications for research studies of the antimicrobial efficacy of ionizing radiation that use nalidixic acid resistance as a marker. This technique was validated for use with chemical interventions and growth parameter measurements (Blackburn and Davies 1994, Taormina and Beuchat 1999) but not for use with ionizing radiation. The wide variation in D_{10} values of the Nal^R versus the parent Nal^S strains would lead to a gross overestimate of the efficacy of ionizing radiation against *E. coli* O157:H7. The calculated dose to achieve a 5 \log_{10} reduction, based on the D_{10} values obtained from the Nal^R isolates in buffer, would actually result in a reduction of only 1.54-2.75

\log_{10} units of the parent Nal^S isolates. The D_{10} values obtained in solution were not predictive of those obtained on lettuce; various isolates were seen to be more, less, or equally sensitive to irradiation (Table 1). In buffer, Nal^R isolates tended to have a more uniform radiation sensitivity than Nal^S isolates; on lettuce, the reverse was observed. This sensitivity to the suspending medium has been noted in a variety of food substrates (Niemira and others 2002; Gombas and others 2003; Niemira 2003), but a complete understanding of the mechanisms by which substrate chemistries influence radiation sensitivity has not been proposed. The overestimation of the efficacy of irradiation based on the results obtained in buffer is therefore only partially mitigated by evaluation of the isolates on the leaf surface rather than in buffer. The 5 \log_{10} dose, based on the lettuce D_{10} values, would actually result in a reduction of 2.78 to 3.29 \log_{10} units. Furthermore, since the magnitude of the increase in radiation sensitivity is variable in an isolate-specific manner, it is not possible to provide a corrective "adjustment factor" to consistently relate the calculated dose based on the Nal^R strains with those of the parent Nal^S strains; this problem would be compounded when multiple isolates are combined in a multi-strain cocktail. The use of Nal^R strains of *E. coli* O157:H7 in studies of ionizing radiation does not provide consistently meaningful information on how wild-type Nal^S strains of *E. coli* O157:H7 would respond to irradiation under real-world conditions.

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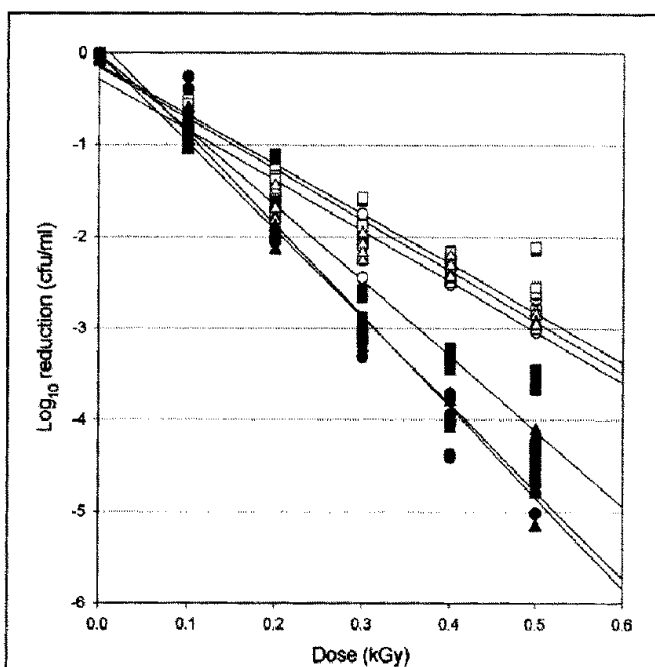


Figure 3—Radiation sensitivity of nalidixic acid sensitive (Nal^S, white) and resistant (Nal^R, black) isolates of *Escherichia coli* O157:H7 on the surface of green leaf lettuce. Isolates C9490 (circle), 35150 (square), and 43894 (triangle) were grown in unamended tryptic soy broth (TSB).